CHAPTER 6

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

In this thesis, we studied the tumor suppressive functions of SETD2 and PBRM1 in ccRCC development. In ***chapter 2***, we comprehensively reviewed the literature concerning SETD2, from its basic biological functions to clinic relevance, especially for ccRCC tumors. In ***chapters 3*** and ***4***, we investigated the consequences of SETD2 and PBRM1 loss in primary tubular epithelial cells (PTECs), the proposed normal counterparts of ccRCC tumor cells. In ***chapter 5***, we broadened our study to long non-coding RNAs (lncRNAs) in an attempt to identify lncRNAs involved in ccRCC development. Here, I summarize our findings, discuss the results in a broader view, and propose some (near-) future perspectives.

SETD2 loss in PTECs

In mammalian cells, SETD2 is the sole protein responsible for the trimethylation of histone H3 at lysine 36 (H3K36me3). The H3K36me3 histone mark is linked to actively transcribed regions. Loss of SETD2 results in loss of H3K36me3, which prohibits binding of H3K36me3 reader proteins to carry out their functions. Consequently, SETD2 deficient cells showed defects in facilitating transcription elongation, preventing spurious transcription initiation, RNA processing, DNA mismatch repair (MMR), and homologous recombination (HR) repair. These defects increase the risk of transformation of SETD2 deficient cells (***chapter 2***). SETD2-loss may also abolish its direct interaction with other proteins, *e.g.* TP53 (Xie et al., 2008). Our current knowledge on the direct binding partners of SETD2 is still limited, which calls for further investigations.

Inactivation of SETD2 prevented PTECs from senescence-induced growth arrest (***chapter 3***), an observation that has not been described before. Interestingly, *SETD2*-knockdown(KD) PTECs retained expression of G2M check-point genes and E2F target genes at a level similar to wild type PTECs at day 6. In contrast, day 16 WT PTECs showed a significant downregulation of these gene sets. Subsequent RT-qPCR showed that the CDKN2A-E2F axis was inhibited in *SETD2*-KD PTECs. In addition, SETD2-loss conveyed PTECs with additional oncogenic expression signatures, *e.g.* genes related to Epithelial-Mesenchymal Transition (EMT). The expression of several lncRNAs was downregulated upon *SETD2*-KD. These downregulated lncRNAs showed further decreased levels in ccRCC cell lines (***chapter 5***). Similarly we also observed a further downregulation of the protein coding gene expression levels in the ccRCC cell lines (***chapter 5***).

The *SETD2*-KD PTECs were insensitive to the normal senescence barrier, a known tumor suppressive mechanism. To our knowledge, this is the first functional study that clarifies how SETD2-loss contributes to ccRCC initiation. The inhibition of the CDKN2A-E2F axis in *SETD2*-KD PTECs caused this senescence resistance. Previously, Xie et al (2008) showed that SETD2 could directly interact with TP53 to modulate a specific set of TP53 downstream genes. Interestingly, we observed an increased expression of *CDKN1A*, the TP53 downstream gene during senescence

induction, in *SETD2*-KD PTECs. Apparently, the activated TP53-CDKN1A axis cannot efficiently establish senescence in *SETD2*-KD cells. After finishing the work reported in the thesis chapters, as a first step to further explore this, we carried out a growth competition assay using lentiviral shRNA constructs against *SETD2* and against *TP53*. This resulted in a mixed culture of untransduced, *SETD2*-KD, *TP53*-KD and double-KD PTECs. We followed the relative abundance of these cells over time. The *TP53*-KD PTECs gradually decreased in abundance much alike the WT-PTECs. This indicates that *TP53*-KD alone does not prevent PTECs from going into senescence. However, the double-KD PTECs showed an evident proliferative advantage over *SETD2*-KD PTECs (Figure 1). This demonstrates that although TP53-loss alone cannot prevent PTECs from going into senescence, it does promote the proliferation of SETD2-deficient cells. These observations are consistent with a study on fibroblasts by Beauséjour et al. (2003) who showed that *CDKN2A* is the second dominant and irreversible factor to establish the senescence barrier after the TP53-CDKN1A axis, and TP53-loss could only induce a robust proliferation in the cells with a low expression of *CDKN2A*. However, It is still not clear how SETD2 mediated H3K36me3 modulates the expression of *CDKN2A* during senescence induction. Several factors could contribute to the decreased expression levels of *CDKN2A* upon loss of H3K36me3, *i.e.* gene body methylation, which is co-localized with H3K36me3 marked regions, and positively associates with gene expression levels (Morselli et

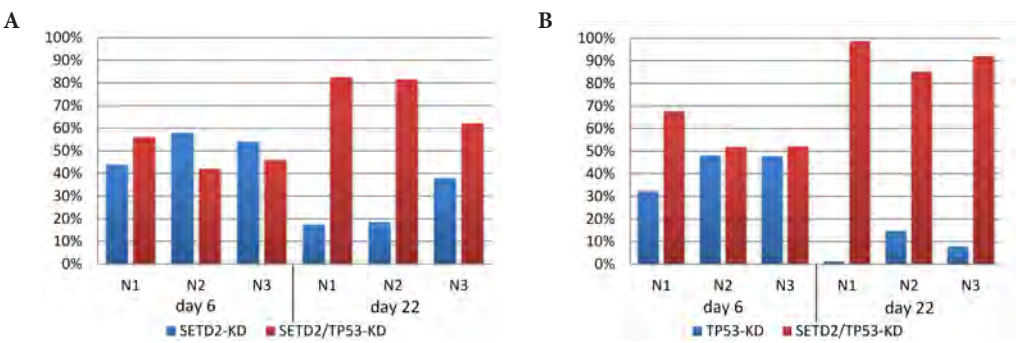

Figure 1. Growth competition data of *SETD2*-KD (A) or *TP53*-KD (B) PTECs with *SETD2*&*TP53*-KD PTEC. PTECs at passage 2 (day 0) were transduced with GFP labeled shRNA against SETD2 and RFP labeled shNRA against *TP53* at low MOI. The percentage of positive fluorescence cells was determined by FACS measurement at indicated time points after transduction. The bars indicate the percentage of positive cells for each cell type. In panel A, the total number of *SETD2*-KD cells is set at 100% for each measurement. In panel B, the total number of *TP53*-KD cells is set at 100% for each measurement. The red bar indicates the percentage of *SETD2/TP53* double knock-down cells at each time point. Data are shown for three independent experiments using three different PTEC cultures. Panel A shows that the double knock-down cells proliferate faster than the *SETD2* knock-down cells. Panel B shows that the *TP53* single knock-down cells have almost disappeared after 22 days.

al., 2015). In addition, the DNA methyltransferases DNMT3 A/B can recognize the H3K36me3 signal through their PWWP domain for DNA methylation (Dhayalan et al., 2010). Thus the absence of H3K36me3 and the subsequent loss of gene body methylation loss may lead to altered chromatin structure of *CDKN2A* gene body, and its decreased expression.

Besides irreversible growth arrest, senescent cells are also characterized by the senescence-associated secretory phenotype (SASP), the secretion of various pro-inflammatory cytokines, chemokines, growth factors and proteases (Campisi J., 2013). Some of the SASP factors are able to activate the immune system to clear senescent cells; while some others promote cell proliferation, angiogenesis and EMT transition. Depending on the context, SASP can be either beneficial or harmful for cancer cells (Campisi J., 2013). We noticed that some of the expression signatures that were enriched in senescent and *SETD2*-KD PTECs, i.e. TNFA_SIGNALING_VIA_NFκB, IL6_JAK_STAT3_SIGNALING and INFLAMMATORY_RESPONSE (***chapter 3***), might be related to SASP. The effect of *SETD2*-KD PTECs on SASP should be further validated at protein level.

H3K36me3 is also present at the body of lncRNA genes. Indeed, H3K36me3 ChIPseq was used to find new lncRNA transcripts (Derrien et al., 2012). It is thus not surprising that *SETD2*-KD PTECs also showed significant changes in the expression levels of lncRNA. Importantly, the downregulated lncRNAs upon *SETD2*-KD were further decreased in ccRCC tumors (***chapter 5***), suggesting that SETD2-loss might also contribute to ccRCC development through changes in lncRNA expression.

SETD2 inactivating mutations are detected in a wide spectrum of tumors, albeit with low frequency. In breast cancer, SETD2 inactivation has been suggested to be one of the driver mutations (Stephens et al., 2012). Our new preliminary data indicate that SETD2 plays a role in the senescence barrier establishment in breast epithelial cells (data not shown). We need to confirm this and it will be attractive to investigate if SETD2 loss will also influence senescence in other primary epithelial cells. To this end, we could perform a stable *SETD2*-KD in a panel of primary cells, especially including the ones assumed to be the normal counterparts of different types of tumors. Studying the growth characteristics of these cells and determine presence of senescence by measuring β-gal activity will indicate whether SETD2 has similar roles in other epithelial cell types. Expression analysis of *CDKN2A* in *SETD2*-KD cells will clarify if SETD2/H3K36 trimethylation is a general mechanism in controlling cellular senescence. It is also worth investigating if SETD2 loss results in alterations of the methylation status of *CDKN2A* gene body. To answer this question, we could perform bisulfite sequencing of the gene body of *CDKN2A* in *SETD2*-KD PTECs. The non-senescent and senescent PTECs, could be included as controls respectively.

The custom designed microarray used in our study also contains both lncRNA probes and protein coding gene probes. This enables us to further identify putative senescence-associated lncRNAs. The lncRNAs that show altered expressions in

senescent PTECs, but remain stable in *SETD2*-KD PTECs, as compared to non-senescent PTECs, are the first candidates to functionally explore. Next, we could determine their abundance in the nuclear and cytoplasmic fractions of the cells respectively. The lncRNAs that are abundant in the nuclear fraction might be relevant for gene expression regulation. Potential cis-regulated target genes could be identified by combining the expression data of lncRNAs and protein coding genes. Through this step-by-step filtering, the number of candidate lncRNAs will be reduced and for this smaller set of candidates, knockdown or overexpression studies could be performed to confirm its function in senescence.

PBRM1 loss in PTECs

In *PBRM1*-KD PTECs we did not observe evident changes in cellular proliferation, or in the process of senescence (**chapter 4**). We did observe significant expression changes (>2 fold) in both protein-coding genes (130 up/155 down) and lncRNAs (9 up/25 down)(**chapters 4 and 5**). For protein-coding genes, the most striking changes for both up and downregulated genes, were related to the IFN-α and IFN-γ responsive gene sets. Both protein-coding genes and lncRNAs with significantly lower expression levels in the *PBRM1*-KD PTECs showed an even lower expression levels in the ccRCC cell lines. These downregulated genes were enriched in gene ontology annotations related to cell differentiation, synapse organization and cytoskeleton organization.

Previous studies on ccRCC cell lines revealed that *PBRM1*-loss promoted the cellular proliferation, migration, and colony formation (Varela et al., 2011). These changes were not observed upon *PBRM1*-KD in PTECs, which are the presumed normal counterparts of ccRCC. This difference might indicate that inactivation of PBRM1 has different roles in ccRCC initiation and progression. Recently, Benusiglio et al (2015) reported an inactivating *PBRM1* germ line mutation in a ccRCC family, of which all identified mutation carriers developed ccRCC tumors. Loss of WT PBRM1 was observed in the tumors. This reinforces the importance of *PBRM1* loss as a driver of ccRCC development.

PBRM1 is one of the subunits specific for the PBAF subgroup of SWI/SNF complexes. The bromodomains of PBRM1 read histones with H3K4 acetylation (H3K4ac), a histone mark enriched at the promoter regions of actively transcribed genes (Wang et al., 2008). In this way, PBRM1 targets the PBAF complex to specific genomic segments to alter the local accessibility of the chromatin. The altered chromatin accessibility subsequently influences expression of the downstream target gene. Thirty-one genes that were differentially expressed upon *PBRM1*-KD also showed altered expression in ccRCC cell lines. Twenty five out of these 31 genes are linked to known biological processes and molecular functions, some of these 25 to multiple processes and functions. Gene ontology annotation revealed presence of 10 genes related to immune response (hormone stimulus response), 6 genes related to chromatin organization and transcription; 6 to cell adhesion; 11 to cellular proliferation and apoptosis.

Aberrant expression of immune response genes upon *PBMR1*-KD of PTECs could contribute to tumor development by facilitating escape from anti-tumor responses (Crusz and Balkwill 2015; Giraldo et al., 2015). Gene Set Enrichment Analysis (GSEA) confirmed involvement of IFN-α and IFN-γ responsive gene sets upon *PBRM1*-KD. Previous studies have already shown that the SWI/SNF complexes are responsible for the expression of IFN responsive genes (Lemon et al., 2001; Liu et al., 2001 and 2002; Huang et al.,2002; Cui et al., 2004; Wang et al., 2004). However these studies did not always pinpoint the precise complex responsible for their findings as they focused on one of the subunits present in all complexes. Our data clearly demonstrate the effect of loss of the PBAF complex on the basal expression of IFN-α/γ responsive genes.

The preliminary data that we collected so far do not fully explain how loss of PBRM1 functionality can be an initiating event in the development of ccRCC. To identify the direct target genes of PBRM1, a chromatin immunoprecipitation (ChIP) sequencing experiment using an antibody against PBRM1 could be considered. Overlapping the ChiP-seq data with the expression data will indicate which genes are the direct *PBRM1*-KD targets. In addition, an assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) can also be used to identify the accessible DNA regions before and after PBRM1-loss.

Our data show that the PBAF complex regulates the basal expression of IFN-α and IFN-γ responsive genes. It will be interesting to investigate if PBRM1 depleted PTECs show different expression of those genes upon IFN-α and/or IFN-γ treatment as compared to their wild-type counterparts. This regulation was investigated in HELA cells in several studies (Lemon et al., 2001; Liu et al., 2001 and 2002; Huang et al.,2002; Cui et al., 2004; Wang et al., 2004). These studies showed that expression of PBRM1 was essential for the expression of IFN responsive genes. Specifically, we could investigate if PBRM1 depleted ccRCC cells show differences in expression levels upon IFN treatment. Subsequently, we should determine if these expression changes are associated with the proliferation status of ccRCC. These results will help us to understand if PBRM1 negative ccRCC cells behave differently from PBRM1 positive ccRCC upon IFN treatment. In addition, these investigations may give clues for understanding why only part of the ccRCC patients respond to immuno-therapeutics, such as interferons (Leibovich et al. 2003, McDermott et al. 2005, Motzer and Molina 2009), and many of these patients developed therapy-resistant tumors after treatment (Sankin et al., 2015). In addition, it will be interesting to investigate if the immuno-treatment resistance is associated with the *PBRM1* mutation status. If so, this could eventually make *PBRM1* mutation status an important therapy-related biomarker.

CcRCC associated lncRNAs

We identified 89 lncRNAs that were significantly differentially (>2 fold) expressed in ccRCC cell lines as compared to PTECs (**Chapter 5**). Several of them also showed altered expression upon *SETD2*-KD and *PBRM1*-KD in PTECs. The downregulated

lncRNAs upon *SETD2*-KD and *PBRM1*-KD showed further decreased expressions in ccRCC derived cell lines. A total of 39 putative lncRNA-protein coding RNA pairs were identified in ccRCC cell lines, 7 pairs in *SETD2*-KD PTECs, and 3 pairs in *PBRM1*-KD PTECs.

Several lncRNAs were reported to be dysregulated in ccRCC tumors, as compared to the non-tumorous tissues (reviewed by Seles et al. 2016). We could only confirm *MEG3* significantly decreased expression in ccRCC cell lines compared to PTECs. Comparisons between previously published microarray data did not show a lot overlap either. This disconcordance is probably caused by the heterogeneous nature of the samples. First, tissue sections are always a mixture of cell populations, containing both tumor cells and other normal cell types. A second reason might be the intra-tumor heterogeneity of the ccRCC tumor itself (Gerlinger et al., 2012). This notion is supported by a study of Malouf et al., who categorized ccRCC tumors into 4 different groups based on their distinct lncRNA expression patterns (Malouf et al., 2015). Probably only a small number of lncRNAs are consistently differentially expressed in ccRCC tumors and cell lines, compared to their normal counterparts.

MEG3 (also known as *GTL2*) was first identified as an imprinted gene located at human 14q (Miyoshi et al., 2000). In a mouse model, *MEG3* has been shown with a dynamic expression pattern during central neural system development (McLaughlin et al., 2006). Cyclic-AMP (cAMP) could facilitate the binding of CREB transcription factors to the promoter region of *MEG3* to regulate its expression, and the methylation of the promoter region could abolish this binding. Decreased expression of *MEG3* was also reported for non-small cell lung cancer. In these cells *MEG3* functions as an inhibitor of proliferation and inducer of apoptosis by upregulating the *TP53* level (Lu et al., 2013). Wang et al. (2015) observed decreased expression of *MEG3* in ccRCC tumors, and its overexpression significantly induced the apoptosis rate in a ccRCC cell line. Both our data and results from other studies indicate that *MEG3* is a tumor suppressive lncRNA that is significantly downregulated in ccRCC tumors.

It is important to further validate the expression levels of the ccRCC-associated lncRNAs that we identified in these cell lines in a panel of tumor samples. To reduce the bias caused by the heterogeneous nature of the tumors, laser microdissection could be used to harvest a homogeneous tumor cell population. Alternatively, RNA fluorescence in situ hybridization can also be used to detect lncRNA molecules in complex tissue samples and identify lncRNA expression directly. To study the functions of selected lncRNAs we could carry out knock-down and knock-in experiments in ccRCC cell lines, followed by monitoring the changes in proliferation, apopotosis and colony formation. These results will help us to understand how lncRNA contributes to ccRCC development.

In addition, the putative cis-acting lncRNA-protein coding gene pairs identified in our study also need further confirmation. This can help us to understand the interactions between lncRNAs and their nearby protein coding genes.

ADDITIONAL FUTURE PERSPECTIVES

Comprehensive understanding of ccRCC initiation

In this thesis, we studied the consequences of SETD2 and PBRM1 loss in PTECs separately, whereas the development of ccRCC is a combination of multiple aberrations. For a comprehensive understanding of ccRCC initiation, we need to study different inactivating combinations in PTECs. The shRNA based approach is limited due to the availability of a limited number of fluorescent detectors. Combination of different inactivating events in a single cell can be achieved by first inducing loss of SETD2, which will allow prolonging culture of these cells and next generate stable knock-out cells by using a clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. The CRISPR-Cas9 has been shown capable of targeting different genomic loci by delivering a single Cas9 enzyme with two or more single guide RNAs (sgRNAs) for DNA cleavage (Kabadi et al., 2014).

CcRCC tumors are characterized by loss of the entire p-arm of chromosome 3. So in order to study the development of ccRCC it would be interesting to mimic this structural aberration in PTECs. To overcome the limited proliferative capacity of primary PTECs, we could first use exogenous hTERT to immortalize these cells. As an alternative to inducing a complete loss of 3p, it might be more feasible to specifically deplete the 3p21 region including the *PBRM1*, *SETD2* and *BAP1* loci within a 5MB region. He et al. (2015) showed the feasibility of this approach by delivering two sgRNAs that target different genomic sites. The resulting double strand DNA breaks causes a genomic deletion of the region flanked by the sgRNAs. With a hemizygous 3p background, introduction of point mutations to the ccRCC tumor suppressor genes can more closely mimic the genetic aberrations occurring in ccRCC tumors. In addition the CRISPR-Cas9 system-mediated genome editing is at the DNA level, which results in a more efficient knockdown. The CRISPR-Cas9 system can also be used for correcting disease-associated genetic aberrations. A relative easy approach may be to repair the mutations in ccRCC cell lines using the CRISPR-Cas9 and monitor phenotypes of the cells. Moving from studies in cell lines to that in animals could help close the gap between observing changes in cell lines that are speculated to lead to cancer at the tissue level and actual ccRCC development. Unfortunately, previous attempts to study ccRCC development in *SETD2* and *PBRM1* knockout mice were unsuccessful (Hu et al., 2010; Zhang et al., 2014; Wang et al., 2004). Both knockouts lead to embryonic lethality, caused by angiogenesis defects ($SETD2^{-/-}$) or cardiac chamber development defects ($PBRM1^{-/-}$). Using a tissue specific promoter, in combination with the Cre/loxP or tetracycline-inducible systems to create inducible kidney epithelial cell specific *SETD2*-KO mice, might overcome this lethal phenotype.

SETD2/H3K36me3 deficient tumor cells might be sensitive to specific treatment approaches. Pfister et al (Pfister et al. 2015) demonstrated that the WEE1 tyrosine kinase inhibitor AZD1775 promotes degradation of ribonucleotide reductase subunit

RRM2 through activation of CDK. The degradation of RRM2 leads to dNTP starvation and subsequent cell death. H3K36me3 facilitates *RRM2* transcription, which implicates that loss of SETD2 dependent H3K36me3 will result in decreased *RRM2* transcription levels. AZD1775 treatment of H3K36me3-deficient tumor cells is therefore expected to result in extremely low levels of RRM2 and subsequently lead to dNTP starvation, S-phase arrest, and apoptosis. Currently, there are more than 20 clinic trials at different phases registered in ClinicalTrails (https://clinicaltrials.gov/) to test AZD1775 efficacy in various tumors.

The SWI/SNF complex is also a promising target for tumor therapy using synthetic-lethal genetic interactions (reviewed by Kaelin (2005)). Synthetic lethality means that an additional loss of function mutation in a gene can specifically kill tumor cells with a specific mutational background. Acute leukemias show defects in transcriptional regulators, i.e. mutations in transcription factors, DNA methylation machinery and so on, but mutations in SWI/SNF subunits are rarely detected. Thus the SWI/SNF complex appears to be important in maintaining the transcriptional program in these cancer cells. It has been shown that loss of BRG1 (a core component of the SWI/SNF complex) could increase apoptosis of leukemia cells, and block cellular differentiation. Meanwhile, BRG1-loss neither influenced the proliferation, nor the viability, of other cancer cells and fibroblasts (Shi et al., 2013), indicating the effect is cell-type specific. Therefore, targeting the SWI/SNF subunits in the tumors with other genetic aberrations may be a possible novel strategy for targeting SWI/SNF mutated tumor samples therapy.

Table 1. Gene ontology analysis.

Gene symbol	GO TERM
BSPRY	ion transport and binding
CCR10	cellular ion homeostasis, chemokine binding
CDA	regulation of cell growth, regulation of nucleotide metabolic process
CFTR	cholesterol metabolic process, response to hormone stimulus
CTSA	intracellular protein transport, peptidase activity
GJB4	gap junction channel activity, channel activity
HIST1H2BD	chromatin organization, DNA binding
IGFBP2	regulation of cell growth, response to hormone stimulus
IL23A	immune response, cell proliferation
ITGB6	inflammatory response, cell-matrix adhesion
KRAS	negative regulation of apoptosis, response to hormone stimulus, positive regulation of NF-kappa B transcription factor activity, positive regulation of MAP kinase activity, Ras protein signal transduction
LOC646626	positive regulation of NF-kappa B transcription factor activity, negative regulation of apoptosis
MMP7	proteolysis, regulation of cell proliferation
NNAT	response to glucose stimulus, regulation of hormone secretion
NT5C3	nucleoside metabolic process
PAPSS1	nucleobase, nucleoside and nucleotide biosynthetic process
PIR	Transcription
PROM1	sensory perception
PRSS8	proteolysis, response to hormone stimulus
PSMB8	mitotic cell cycle, immune response
RMI2	DNA metabolic process, DNA replication
S100A4	epithelial to mesenchymal transition, calcium-dependent protein binding
SAT1	N-acetyltransferase activity
TNC	cell adhesion
TNF	immune response, positive regulation of NF-kappaB transcription factor activity, cell adhesion, negative regulation of apoptosis

Note: 31 genes, differentially expressed in both PBRM1-KD PTECs and ccRCC cell lines as compared to WT PTECs, were included in the analysis. The genes annotated in the DAVID resource (see chapter 4) with the GO terms 'biological process' (GOTERM_BP_FAT) and 'molecular function' (GOTERM_MF_FAT) are presented in this table.

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